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Spermiogenesis and Sperm Structure in Relation to Early Events of Fertilization in the Limpet *Tectura testudinalis* (Müller, 1776)

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Abstract. Spermiogenesis and fertilization in the limpet *Tectura testudinalis* (Mollusca: Archaeogastropoda: Patelloidea) were examined using scanning and transmission electron microscopy, as well as light microscopy. Spermiogenesis was similar to that described for other Lottiidae (Patelloidea), but some key differences were noted.

We observed that the acrosomal vesicle forms on the plasma membrane from several Golgi-derived proacrosomal vesicles during the early spermatid. Evidence from anti-actin antibody staining of a related species, *T. scutum,* indicates that the acrosome probably contains G-actin in three different locations, but there are no preformed actin filaments. The flagellum of mature sperm tapers to a thin, filamentous end-piece.

The mature egg of *T. testudinalis* is enclosed by a thin vitelline layer and a thick jelly coat bound by follicle cells. Where a mature sperm encounters the intact jelly coat, its acrosome tip may elongate, up to twice its original length. The acrosome reaction is not induced, but by this mechanism the acrosome bridges the jelly coat and extends down far enough to contact the vitelline layer. During the elongation of the acrosome, microfilaments are formed inside the tip. As well, new membrane required for this process is supplied by elimination of any slack in the plasma membrane and also perhaps by spontaneous formation of vesicles inside the acrosome. Once the tip of the sperm contacts and binds with the vitelline layer it undergoes the acrosome reaction, in which the plasma and acrosomal membranes fuse and roll back in a manner typical of scaphopods and some polychaetes. Sperm also may bind directly with the vitelline layer when the jelly coat is absent. Furthermore, the acrosome tip may undergo spontaneous fusion with a naked egg microvillus that formerly connected with a follicle cell process. Thus, the sperm either individually or collectively are equipped to deal with any one of several potential routes to successful fertilization of the egg.

Current theories of gastropod phylogeny place the limpets as basal to the Gastropoda, but new evidence presented here supports the idea that they are a divergent group, with some unique innovations in sperm design.

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Introduction

The process of fertilization in invertebrates typically involves intricate structural responses of both sperm and egg; these are often unique to a particular species and function to regulate sperm-egg recognition as well as sperm entry into the egg (see reviews by Dan, 1967; Colwin and Colwin, 1967; Epel, 1978; Monroy and Rosati, 1983; Tilney, 1985; Koch and Lambert, 1990; Buckland-Nicks, 1995). These mechanisms, often interdependent, work in conjunction to facilitate sperm penetration of the outer protective envelopes of the egg prior to a successful fertilization (Tilney, 1985).

In free-spawning marine invertebrates, ripe unfertilized eggs have one or more extracellular envelopes. In some groups these egg envelopes play a key role in the induction of the sperm acrosome reaction, sperm-egg binding, and the exclusion of supernumerary sperm (see reviews by Colwin and Colwin, 1967; Epel and Vacquier, 1978; Monroy and Rosati, 1983; Tilney, 1985; Longo, 1987; Sato and Osanai, 1990). Just as the egg plays a role in controlling sperm penetration, the sperm itself undergoes a complex series of chemical (exocytotic) and morphological changes that permit penetration of the egg envelopes

and fusion with the egg membrane. Classic studies on echinoderms have shown that contact with the egg jelly coat induces exocytosis of the acrosomal vesicle, which releases lysins that dissolve the egg envelopes (see reviews by Colwin *et al.,* 1975; Tilney, 1985; Epel and Vacquier, 1978). Simultaneous with the exocytosis of lysins is the rapid extension of an acrosomal process that projects the inner acrosomal membrane into contact with the vitelline layer overlying the egg microvilli. Typically this independent process involves either the rapid polymerization of globular to filamentous actin to form a rod-like structure (Schroeder and Christen, 1982; Tilney, 1985), or the inter-sliding of actin filaments in a preformed rod, causing its extension, as in bivalves (Tilney, 1985; Tilney *et al.,* 1987) and some archaeogastropods (Lewis *et al.,* 1980; Shiroya *et al.,* 1986; Shiroya and Sakai, 1993). Species-specific recognition and binding with the vitelline layer and its subsequent dissolution by other acrosomal lysins results in fusion when specific receptors on the inner acrosomal membrane contact their counterparts on the egg membrane.

The exocytosis of acrosomal lysins and the extension of an acrosomal process (or its equivalent as in chitons [Buckland-Nicks *et al.,* 1988]) are typical of mechanisms used to penetrate the egg envelopes of free-spawning marine invertebrates; however, they may not always be necessary even when present (see discussion in Monroy and Rosati, 1983). In some species with reduced or absent jelly coats, the evagination of the inner acrosomal membrane may not require an acrosomal process, or if so, a very reduced one (Dufrêsne-Dubé *et al.,* 1983; Sato and Osanai, 1983, 1990).

Among molluscs, studies of early events of fertilization have largely been restricted to species with preformed axial rods, such as bivalves (Niijima and Dan, 1965; Hylander and Summers, 1977; Tumboh-Oeri and Koide, 1982; Longo, 1973, 1987), and some archaeogastropods (Lewis *et al.,* 1980; Sakai *et al.,* 1982; Shiroya and Sakai, 1984, 1992; Shiroya *et al.,* 1986, 1989). In other gastropod molluscs, these early events are virtually uncharted. This is partly because many gastropods fertilize internally and the conditions, which involve capacitation of the sperm as well as specific changes to the egg, are difficult to simulate in the laboratory. Thus, external fertilization was an important consideration in our selection of the limpet *Tectura testudinalis* (Gastropoda: Patelloidea: Lottiidae) as a suitable model in which to study early events of fertilization.

The Patelloidea are of particular interest in light of the ongoing debate as to their phylogenetic status among 'Archaeogastropoda' *sensu lato* (Branch, 1985; Haszprunar, 1988; Hodgson, 1995; Lindberg, 1988; Buckland-Nicks and Scheltema, 1995; Hodgson *et al.,* 1996). Sperm structure has recently been found to provide a series of characters useful for testing extant hypotheses on phylogenetic relationships (Healy, 1988, 1996; Giusti *et al.,* 1990; Justine, 1991; Jamieson *et al.,* 1991; Jamieson, 1991) and is one of the main reasons for concluding that in several animal groups external fertilization with "primitive" sperm is not necessarily primitive (Jamieson, 1991; Rouse and Fitzhugh, 1994; Buckland-Nicks, 1994). Rather, internal fertilization with introsperm may be basal to the Bilateria (Buckland-Nicks and Scheltema, 1995). Justine (1991) emphasized the importance of studying early events of spermiogenesis to obtain a complete understanding of how sperm organelles are formed. There have been many studies of mature sperm ultrastructure in Patelloidea (Azevedo, 1981; Kohnert and Storch, 1983; Koike, 1985; Smaldon and Duffus, 1985; Hodgson and Bernard, 1988, 1989; Jamieson *et al.,* 1991; Hodgson and Chia, 1993; Sousa and Oliveira, 1994; Hodgson *et al.,* 1996), but only one detailed study of spermiogenesis (Hodgson and Bernard, 1988).

What has been overlooked so far is that a number of taxon-specific changes in sperm ultrastructure occur during the early events of fertilization. Thus, the study of fertilization, which is intrinsically interesting, can also provide new characters for improving the accuracy of current hypotheses on phylogenetic relationships. Our detailed description of spermiogenesis in *T. testudinalis* broadens the base of knowledge and provides new morphological characters for use in phylogenetic analysis of the Archaeogastropoda. Furthermore, it documents, for the first time in a limpet, the basic process of sperm-egg interaction—which turns out to be surprisingly unusual.

Materials and Methods

Spermiogenesis and mature sperm ultrastructure

Specimens of *Tectura testudinalis* (Müller, 1776) (see Lindberg, 1986) were collected from the intertidal regions at Port Bickerton, Nova Scotia, between June and September 1994. *T. testudinalis* is gonochoric (dioecious), and males and females were sorted on the basis of ventral foot coloration. In the male, a whitish band corresponding to the testis is visible on the left underside of the foot, which corresponds to the right (gonadal) side of the limpet. In the female, this same region is reddish. *T. scutum* (Rathke, 1833) was collected from the intertidal zone on San Juan Island, Washington, in August 1995 and was used only to study actin distribution in the acrosome (see Epifluorescence microscopy, this section).

Small portions of the male testis were prepared for transmission electron microscopy (TEM) by excising testis from the body mass, mincing it into pieces of about 1 mm^3, fixing in ice-cold 2.5% glutaraldehyde (brought to pH 8.0 using NaOH), in Millipore-filtered seawater (MFSW), and allowing the tissue to come to room temper-

ature overnight. The primary fixative was removed, and specimens were washed in 0.2 *M* sodium cacodylate buffer (pH 7.6) for 10 min, then transferred to ice-cold 1.25% OsO_4 in 0.2 *M* sodium cacodylate buffer (marine rinse) as a secondary fixative for 1 h. Subsequent washing in 0.2 *M* sodium cacodylate buffer for 15 min was followed by dehydration in a graded ethanol series (30, 50, 70, 80, 90, 95, 100 × 3), for 10 min in each concentration. Pieces were then infiltrated in 1:1 Spurr's: 100% ethanol mixture and left overnight in a desiccator.

The following day, specimens were placed in 100% Spurr's resin for 6–8 h, transferred to dried polyethylene BEEM capsules containing fresh Spurr's medium, and baked overnight at 70°C. Polymerized blocks were removed from the capsules, rough trimmed, and semi-thin sectioned with a glass knife mounted in a Sorvall MT-2 ultramicrotome. Thick sections were stained with Richardson's stain. When an area of the egg was encountered with many juxtaposed sperm, suggesting polyspermy, the block was thin sectioned with a diamond knife (Diatome) mounted in a Sorvall MT-2C ultramicrotome. Sections with silver-gold interference color were picked up on naked 150-mesh copper grids, stained sequentially with saturated aqueous uranyl acetate for up to 30 min followed by lead citrate for 10 min, and examined in a Philips 300 transmission electron microscope.

Early events of fertilization

Preparation of gametes. Ripe animals from the field collections were "stripped" to obtain gametes. The entire gonad was cut away from the gut and body mass of both the males and the females and placed into separate clean petri dishes. The ovary was cut open and gently prodded with a blunt pointer while being flushed with 0.45-μm MFSW to expel eggs. Eggs were transferred in clean MFSW to a small centrifuge tube and gently centrifuged to separate any tissue debris into the supernatant, which was removed and replaced with fresh MFSW.

The testis was macerated dry to expel some sperm concentrate as well as to reduce premature activation by preventing contact with seawater. Sperm concentrate could be stored in the refrigerator in sealed Eppendorf vials for 1 or 2 h or used immediately by gently drawing several drops into a Pasteur pipette and adding them to a vial containing 10 ml of MFSW to activate them. This activated sperm concentrate was used within 10 min for fertilization of eggs.

Fertilization. In an attempt to induce polyspermy for microscopic study, activated sperm concentrate was added to a portion of washed eggs in a small vial and mixed well by gentle agitation. Mixing by pipetting was avoided because it damages sperm acrosomes. Fertilized eggs were fixed and dehydrated as previously described for gonads. Fertilization success, as determined by verifying that fertilized eggs underwent cleavage, was considerably improved by prior treatment of eggs with 5-hydroxytryptamine (5HT) (Juneja *et al.,* 1993) and only slightly improved by pretreatment with alkaline seawater (pH 9.0) (Lewis *et al.,* 1980). Eggs pretreated with 5HT also showed some improvement in apparent polyspermy when subjected to sperm concentrates. Batches of eggs that were treated with Hoechsts 33342 DNA stain were examined with UV light in an Olympus BH2 light microscope equipped with epifluoresecence. If many sperm nuclei appeared juxtaposed with the egg membrane, indicating polyspermy, the rest of that batch would be fixed for electron microscopy, as before, with the following modifications for scanning electron microscopy (SEM).

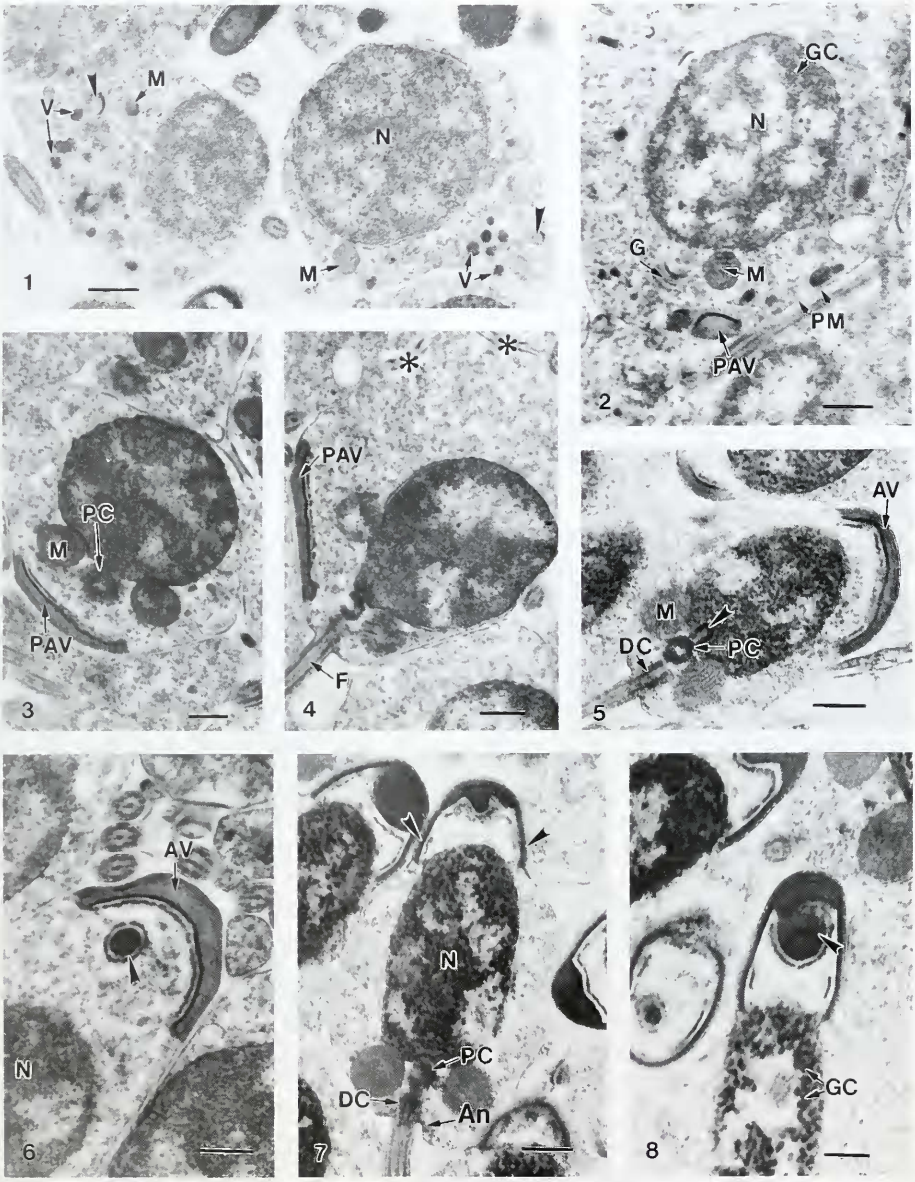
Scanning electron microscopy. Following dehydration, fertilized eggs were critical-point dried (Samdri) from 100% ethanol. The dried eggs were mounted on "JEOL" or "Cambridge" aluminum stubs with double-sided carbon sticky tape (Pelco). The stubs were coated with gold in a Polaron SC502 sputter coater and examined using a JEOL 5300 scanning electron microscope operated at 15 kV.

Epifluorescence microscopy. Samples of sperm of *T. scutum* were fixed for 30 min in 4% paraformaldehyde in 0.45-μm MFSW and mounted on coverslips coated with poly-L-lysine (Sigma #A-4700). The coverslips were rinsed in PBS (phosphate buffered saline) three times and incubated for 1 h in anti-actin (primary) antibody developed in mouse (1/200 dilution) (Sigma). Coverslips were washed three times with PBS (15 min each wash) and incubated in rabbit anti-mouse IgG conjugated to FITC (1/200 dilution) (Sigma #F-7506) for 1 h. Coverslips were again washed three times in PBS and then mounted in PBS in glycerol with DABCO as an antibleaching agent. Controls were treated the same except that the primary antibody was omitted. Permanent slides were made by sealing stained coverslips to slides with "Wet and Dry" nail varnish. Prepared slides were examined in a Nikon Optiphot 2 fluorescence light microscope fitted with FITC filters. Micrographs were taken with TMax 400 film (Kodak) exposed at 800 ASA and developed for normal times in D76 developer (Kodak).

Results

Spermiogenesis

In early spermiogenesis, the Golgi body secretes numerous vesicles of varying density, some of which fuse to form a proacrosomal vesicle (Fig. 1), which becomes ovoid and attaches to the plasma membrane by some granular material (Fig. 2). Electron-dense material develops on the intracellular side of the proacrosomal vesicle as it enlarges and elongates (Figs. 2–4). While the Golgi



Figures 1–8. Transmission electron micrographs of spermiogenesis in *Tectura testudinalis*.

Figure 1. Early spermatid with large spherical nucleus (N), small mitochondria (M), and dense Golgi-derived vesicles (V) located at presumptive posterior of cell adjacent to small proacrosomal vesicle (arrowheads). Scale bar = 0.5 μm.

body remains basal, the maturing acrosomal vesicle migrates along the plasma membrane to the apex of the nucleus (Figs. 4, 5) and becomes cup-shaped with the concave side facing the nucleus (Figs. 5–8). Later in development, an invagination of the inner acrosomal membrane expands to form the posterior lobe (Fig. 8). This posterior lobe extends into the subacrosomal space (Fig. 9) and continues to elongate until it abuts some flocculent material on top of the nucleus (Fig. 10). Sometimes sections show a separate spherical vesicle in the subacrosomal space (Fig. 6), but this is interpreted as a cross section of the developing posterior lobe.

Early in spermiogenesis, many small mitochondria are distributed throughout the cell (Fig. 1). As the spermatid matures, the mitochondria aggregate posteriorly at the base of the nucleus (Figs. 5, 7), and eventually fuse to form a ring of four spherical mitochondria enclosing the proximal and distal centrioles (Figs. 5, 7, 11). The proximal centriole lies perpendicular to the apex of the distal centriole and becomes attached by fibrogranular material to a posterior invagination of the nucleus, termed the nuclear fossa (Figs. 5, 7). Extending posteriorly from the distal centriole is the elongating flagellum (Fig. 4).

The large spherical nucleus of the early spermatid comprises heterogeneous aggregations of granular chromatin (Figs. 1, 2). As the spermatid matures, the nucleus elongates in the anterior-posterior axis and the chromatin condenses to a coarse granular appearance (Figs. 7, 8). The cytoplasm of the developing spermatids in a cohort is joined by cytoplasmic bridges (Fig. 4), which are eliminated together with the residual cytoplasm when sperm are liberated into the lumen.

Ultrastructure of mature sperm

Mature sperm have an overall length of about 42 μm. The sperm are divided into four regions: head, mid-piece, principal-piece, and end-piece. The head region comprises a conical acrosome measuring about 3.1 μm $\times$ 0.7 μm and, posterior to this, a bullet-shaped nucleus of 2.6 μm $\times$ 1.0 μm, slightly tapered towards the apex (Figs. 10, 14). The total length of the head region is 5.7 μm. The mid-piece is 1.0 μm in length, comprising a ring of four mitochondria enclosing the centriolar complex, and includes the annulus in a "collar" encircling the flagellum (Fig. 14). The principal piece and end-piece have an overall length of 35.0 μm, of which the end-piece constitutes 5.2 μm.

The elongate acrosome cone exists as a two-part structure comprising an anterior portion, 1.4 μm in length, and a posterior portion containing the subacrosomal space, 1.2 μm in length (Fig. 10). The anterior acrosome is characterized by a layering of electron densities and granularities (Fig. 10). A central invagination of the inner acrosomal membrane extends posteriorly as a lobe into the subacrosomal space and abuts flocculent material at the anterior tip of the nucleus (Fig. 10 and inset). No microfilaments were visible within the lobe or the anterior acrosome. The subacrosomal space consists of a less electron-dense material that lacks the stratification of the anterior acrosome, but houses the invaginated posterior lobe.

The nucleus comprises homogeneously dense chromatin with some lacunae and lies in a small posterior fossa that houses the centrioles (Fig. 10). The mid-piece is located posterior to the nucleus and consists of a ring of four spherical mitochondria surrounding the centrioles (Figs. 11, 14). The proximal centriole is oriented perpendicular to the distal centriole, from which emanates the axial flagellum. A centriolar satellite complex comprised of nine spoke-like satellites connects the distal centriole with the annulus, which is attached to the plasma membrane, later forming a distinct collar posterior to the mid-piece (Fig. 14). The flagellum has a conventional 9 + 2

Figure 2. Early spermatid with aggregations of granular chromatin (GC) in nucleus (N). Some dense vesicles secreted from Golgi body (G) have fused posteriorly to form an ovoid proacrosomal vesicle (PAV), which is attached to plasma membrane (PM). Mitochondrion (M). Scale bar = 0.5 μm.

Figure 3. Spermatid with oblong proacrosomal vesicle (PAV) located posteriorly in cell. Mitochondrion (M), proximal centriole (PC). Scale bar = 0.5 μm.

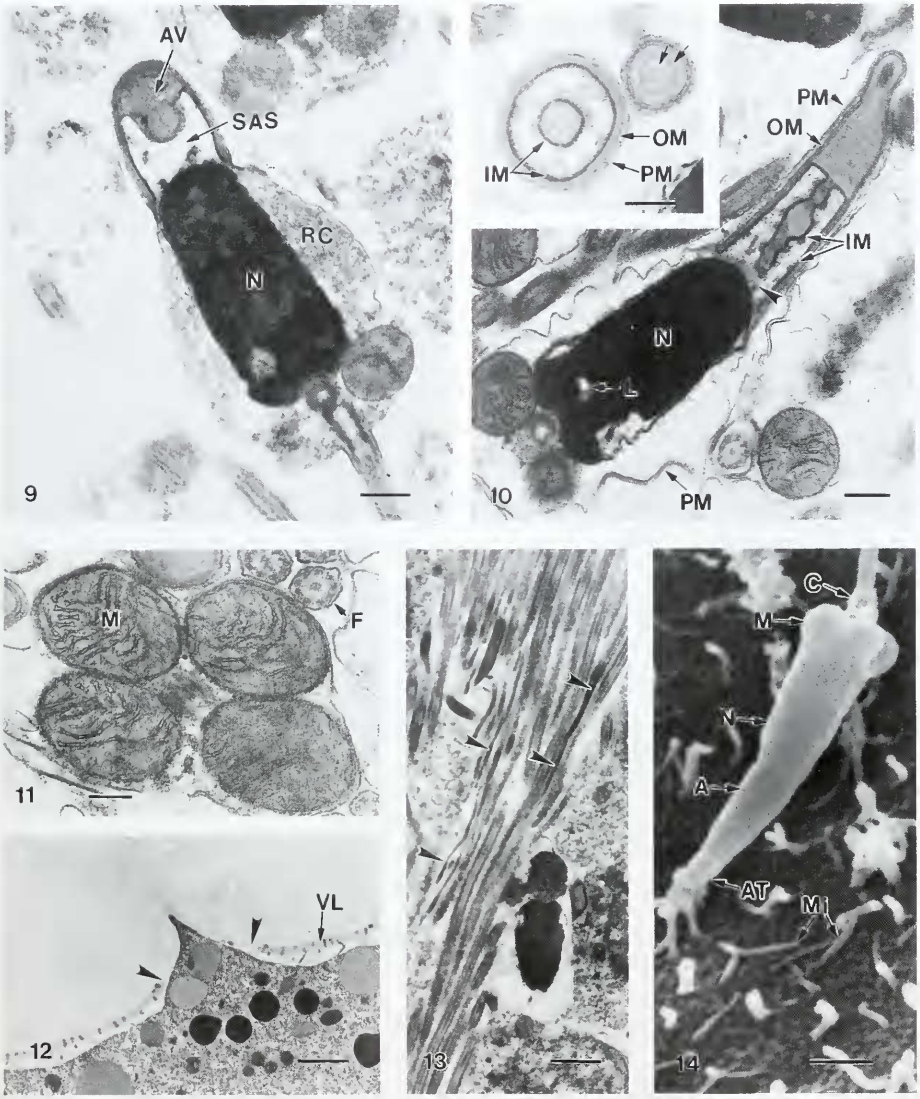
Figure 4. Longitudinal section of spermatid with proacrosomal vesicle (PAV) migrating to presumptive anterior of cell. Note Flagellum (F) and cytoplasmic bridges (*) connecting adjacent spermatids of a cohort. Scale bar = 0.5 μm.

Figure 5. Spermatid acrosomal vesicle (AV) with cup-shaped morphology at apex of nucleus. Proximal centriole (PC), distal centriole (DC), mitochondrion (M), and electron-dense nuclear fossa (arrowhead). Scale bar = 0.5 μm.

Figure 6. Cup-shaped acrosomal vesicle (AV) above nucleus (N) with circular profile of possible small proacrosomal vesicle (arrowhead) in subacrosomal space. Scale bar = 0.4 μm.

Figure 7. Later spermatid than in Fig. 6, showing elongating nucleus (N) and lateral extension of acrosomal vesicle (arrowheads). Proximal centriole (PC) is attached to nuclear fossa; distal centriole (DC) is harnessed to plasma membrane by the annulus (An) via the centriolar satellite complex. Scale bar = 0.5 μm.

Figure 8. Spermatid showing acrosomal vesicle forming posterior lobe (arrowhead). Note condensation of granular chromatin (GC) into large granules. Scale bar = 0.5 μm.



Figures 9–14. Electron micrographs of sperm and egg structure in *Tectura testudinalis*.

Figure 9. Longitudinal section of late spermatid. Acrosomal vesicle (AV) extending posterior lobe into subacrosomal space (SAS). Residual cytoplasm (RC) next to nucleus (N) will be shed when sperm matures. Scale bar = 0.4 μm.

Figure 10. Longitudinal section through mature sperm showing plasma membrane (PM) enclosing outer (OM) and inner (IM) acrosomal membranes. Note nucleus (N) with lacuna (L), acrosome with layered contents and subacrosomal flocculent material (arrowhead). Scale bar = 0.4 μm. **Inset:** Transverse sections of acrosome near tip, showing electron-dense stratifications (small arrows) and through subacrosomal space showing outer (OM) and inner (IM) acrosomal membranes enclosed by plasma membrane (PM). Scale bar = 0.3 μm.

arrangement of microtubules in the principal-piece (Fig. 11) and tapers into a filamentous electron-dense end-piece (Fig. 13). These observations are summarized diagrammatically in Figure 27.

Surface ultrastructure of unfertilized egg

Intact unfertilized eggs are characterized by two distinct egg envelopes (Figs. 15–18). The outermost envelope is a discontinuous jelly coat about 3 μm thick. This jelly coat surrounds a very thin (0.3 μm), transparent vitelline layer that overlies the plasma membrane. Numerous microvillous extensions of the egg plasma membrane (0.3 μm or more in length) are embedded in the vitelline layer, resulting in small bumps or protrusions of the vitelline layer above the egg surface (Figs. 17, 24–26). Moreover, spaced regularly over the egg surface are a series of much larger, more elongate microvilli (up to 3 μm in length), which penetrate through the vitelline layer and extend up into the egg jelly coat (Fig. 15, 16). Follicle cells overlying the egg jelly coat are connected to the oocyte by microvillous extensions that form junctions with the elongate egg microvilli (Fig. 16). These connections appear at regular intervals over the egg surface (Fig. 15) and appear to disrupt the vitelline layer at these points (Fig. 12). In some areas where the follicle cell processes have retracted, the egg jelly diffuses into the surrounding medium (Figs. 15, 17). Thus, the egg jelly coat of freshly spawned eggs is sometimes discontinuous, where it has dissolved between retracted follicle cells. Furthermore, the jelly coats of adjacent eggs may become stuck to one another, producing small aggregates of eggs.

Early events of fertilization

Mature sperm, in attempts to fertilize the egg, often get lodged in the thick jelly coat (Figs. 18, 18 inset). Sperm in contact with the jelly coat do not undergo an acrosome reaction involving exocytosis of the acrosomal vesicle, but in many instances they do undergo an elongation of the anterior portion of the acrosome (Figs. 18, 19, 22). The elongated acrosome tip is characterized by longitudinal microfilaments, which were not present before elongation (Figs. 19, 19 inset). Posterior to the elongating tip in the middle and base of the acrosome, there appear small membranous vesicles (Fig. 19). During this elongation, the ring of spherical mitochondria usually become compacted into more oblong structures (Figs. 25, 26).

Upon penetration of the jelly coat, the tip of the acrosome comes in contact with the vitelline layer. Contact with the vitelline layer overlying the microvilli initiates the typical acrosome reaction, in which the outer acrosomal membrane and plasma membrane fuse at the apex and roll back in a manner that releases the contents of the acrosomal vesicle onto the vitelline layer (Figs. 25, 26). Following this, the posterior lobe of the inner acrosomal membrane everts and protrudes from within the acrosome (Figs. 25, 26). It is probably during this process that the vitelline layer is dispersed and the inner acrosomal membrane is brought into contact with one or more exposed microvilli of the egg plasma membrane. However, primary contact may also occur between the larger, naked egg microvilli that formerly connected with the follicle cell processes (Figs. 24, 25).

Contact between a naked egg microvillus and the inner acrosomal membrane results in fusion and the creation of a tube through which the nucleus and other sperm organelles gain access to the egg cytoplasm. Following this initial fusion, other microvilli become attached to the inner acrosome, including the larger microvilli that formerly anchored the follicle cells (Fig. 25). The combined fusion of these microvilli results in the production of a fertilization cone, which engulfs the fertilizing sperm. Cortical granules in the cortex of the egg are released following sperm-egg fusion and presumably are involved in a block to polyspermy, as in other invertebrates. On several occasions, follicle cells that had phagocytized supernumerary sperm were found on the surface of fertilized eggs (Fig. 23).

Discussion

Spermiogenesis and ultrastructure of mature sperm

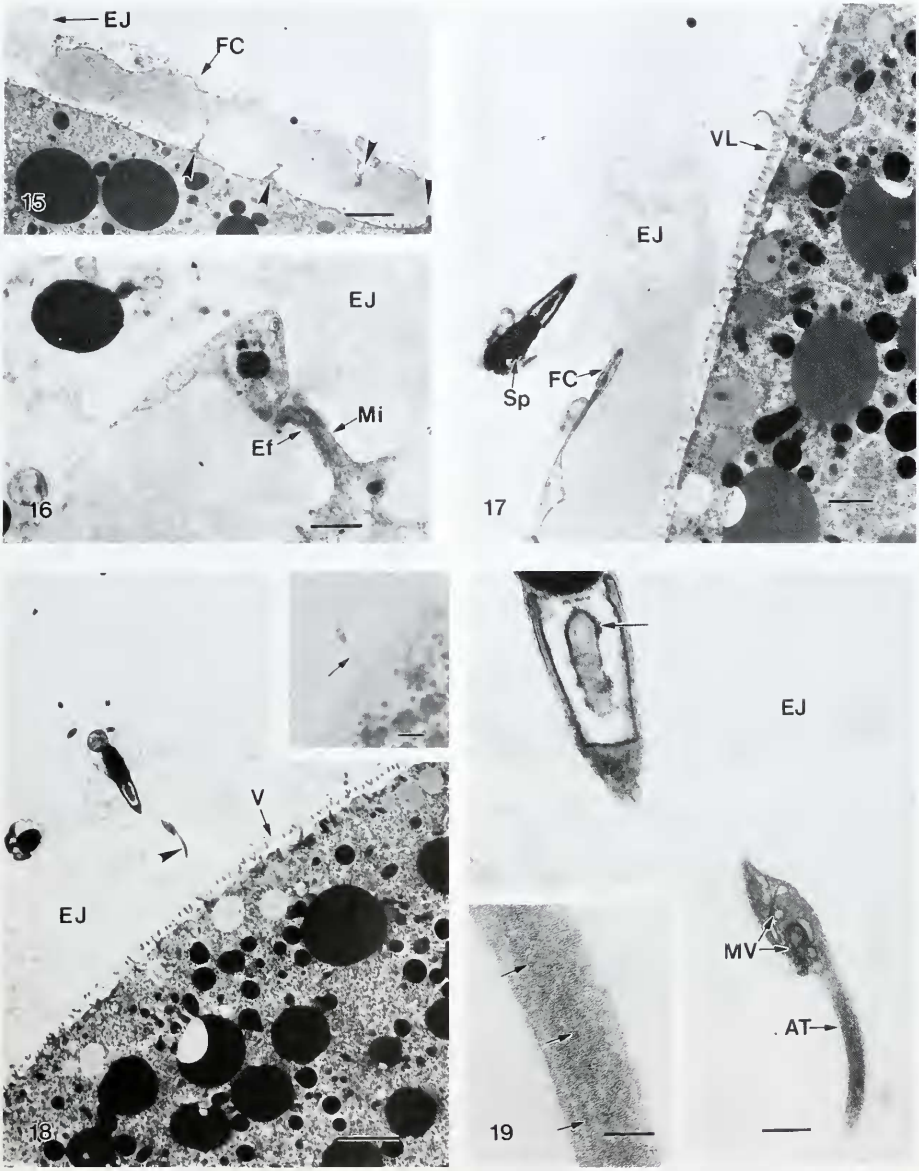
The structure of the unreacted acrosome is similar to that described for some Patellidae (Hodgson and Bernard,

Figure 11. Transverse section of mid-piece of mature sperm showing ring of four spherical mitochondria (M). Note adjacent flagellum (F) with 9 + 2 arrangement of microtubules. Scale bar = 0.2 μm.

Figure 12. Section perpendicular to egg surface showing portion of elongate microvillus penetrating otherwise intact vitelline layer (arrowheads) to make connection with follicle cell process (not shown). Scale bar = 2.0 μm.

Figure 13. Longitudinal section through flagella tapering to electron-dense filamentous end-pieces (arrowheads). Scale bar = 1.0 μm.

Figure 14. Scanning electron micrograph of mature sperm. Note relative proportions of mature sperm components, including acrosome (A) with distinct tip (AT), nucleus (N), spherical mitochondria (M), and collar (C) surrounding flagellum. Note few large egg microvilli (Mi) that formerly bound the follicle cells above the jelly coat; many small microvilli beneath vitelline layer make pattern of bumps on egg surface. Scale bar = 1.0 μm.



Figures 15–19.

Figure 15. Follicle cells (FC) connected to oocyte membrane at regular intervals over egg surface (arrowheads). Note dissipation of egg jelly coat (EJ). Scale bar = 3.0 μm.

Figure 16. Interdigitation of elongate microvillus (Mi) from oocyte membrane with extension of follicle cell (Ef) within egg jelly coat (EJ). Scale bar = 1.0 μm.

1988; Hodgson, 1995) but is closest to that of the Lottiidae (Hodgson and Chia, 1993), all of which are characterized by an acrosomal vesicle with an invaginated posterior lobe that protrudes into the subacrosomal space. However, in *Helcion*, preformed microfilaments in the anterior acrosome project down into the posterior lobe, deep into the subacrosomal space (Hodgson and Bernard, 1988). These microfilaments are unusual in having a diameter of 9 nm, which neither matches actin (6 nm) nor intermediate filaments (10 nm) in size. Similarity in sperm structure between species of *Lottia* and *Tectura* provides support for Lindberg's (1986) reclassification of all *Acmaea* (except *A. mitra*) and all *Notoacmea* (except Australian and New Zealand species) into the genus *Tectura* within the Lottiidae. The structural changes that occur during spermiogenesis in *Tectura testudinalis* are similar to those described for other Lottiidae (Hodgson and Bernard, 1988; Sousa and Oliveira, 1994; Hodgson, 1995). However, the formation of an acrosomal vesicle from fusion of smaller Golgi-derived vesicles, confirmed in this study, has been demonstrated previously only in *Helcion pellucidus* (Sousa and Oliveira, 1994). Moreover, in our study, proacrosomal vesicle formation occurs in the spermatid stage, whereas Sousa and Oliveira (1994) report its occurrence in the primary spermatocyte. In the paper by Sousa and Oliveira (1994), cells described as primary spermatocytes appear to be spermatids, based on the appearance of chromatin (figures 3 and 4) and the position and presence of centrioles associated with mitochondria (figure 4). Regardless, this type of proacrosome formation is consistent with similar processes reported for other archaeogastropods (Azevedo *et al.*, 1985; Koike, 1985; Buckland-Nicks and Chia, 1986) and bivalves (Longo and Dornfeld, 1967), and it is probably present throughout the Patelloidea. It contrasts with the development of the acrosome in caenogastropods, and other invertebrates, from a single cisterna of the Golgi body which remains connected throughout development (Buckland-Nicks and Chia, 1976; Healy, 1982; Koike, 1985).

The typical flagellum of *T. testudinalis* tapers to a filamentous end-piece less than one-third of its diameter. This has only once been reported in Patelloidea (Azevedo, 1981), but it also occurs among Aplacophora and Polyplacophora (Buckland-Nicks, 1995), as well as some Caenogastropoda (Giusti and Mazzini, 1973). It is possible that the filamentous end-piece has not been noticed previously in other patellids and archaeogastropod groups, and may be a useful character in phylogenetic analysis.

Surface ultrastructure of unfertilized eggs

The discontinuous nature of the jelly coat of some *T. testudinalis* eggs suggests that its role is not to act as a sperm barrier, nor to induce the typical acrosome reaction in which the acrosomal vesicle releases its contents, but rather to serve some other role in producing successful fertilization. The egg jelly coat of some keyhole limpets has been shown to contain sperm-activating agents (Tyler and Fox, 1940). Retraction of the follicle cells, which exposes the egg jelly coat in *T. testudinalis*, may result in the release of sperm-activating and sperm-attracting substances as the egg jelly coat dissipates. In thick sections, a relatively high number of sperm were found associated with egg jelly coat bursts from the follicle cell layer.

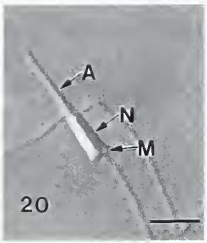
The egg jelly coats of neighboring eggs often stick together to produce an aggregate of eggs. Similar aggregates have been noted in other molluscan groups (Eernisse, 1988; Buckland-Nicks, 1995). Studies by Levitan (1993) on echinoderms have shown that larger eggs may present a larger target for sperm and thus become fertilized at a higher rate. Perhaps aggregates of eggs mimic a larger egg target. Selective pressure would favor such aggregates if they resulted in relatively higher fertilization rates than for individual eggs.

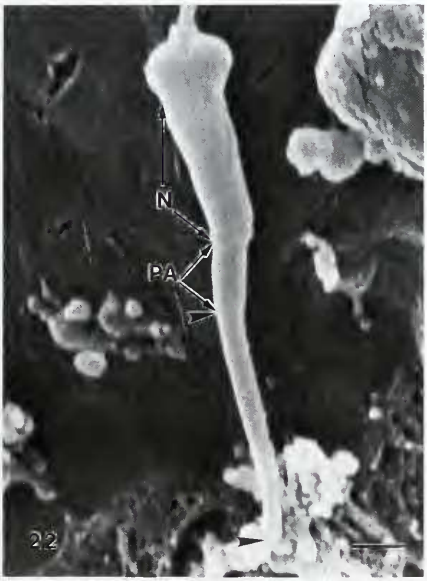
It was surprising to find that follicle cells had assumed a phagocytic function on the surface of fertilized eggs. However, on several occasions, sperm were found inside follicle cells in an obvious state of degeneration. Do waste sperm in some way pose a threat to the zygote? What is the fate of these follicle cells? In other molluscs, such as chitons, the follicle cells are shed before spawning or soon afterwards, because in many species they cover receptive sites on the egg hull (Buckland-Nicks, 1995).

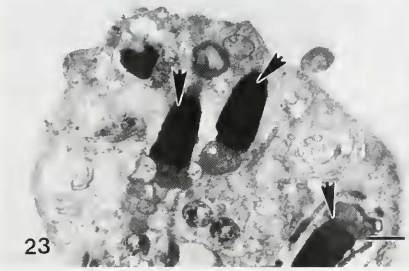
Figure 17. Section of egg showing approaching sperm (Sp), dissipating egg jelly coat (EJ), and vitelline layer (VL) overlying plasma membrane. Note retracted follicle cell process (FC). Scale bar = 1.5 μm.

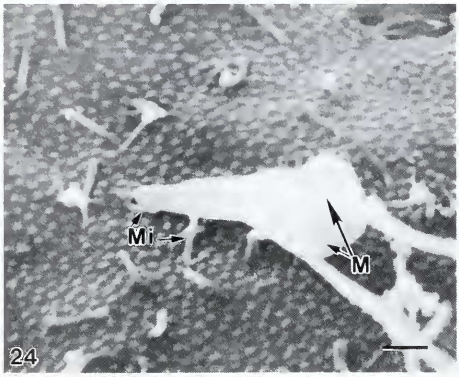
Figure 18. Transmission electron micrograph showing sperm in egg jelly coat (EJ). Note thin vitelline layer (V) and elongated anterior tip of acrosome (arrowhead) belonging to adjacent sperm. Scale bar = 3 μm. **Inset:** Light micrograph of 1-μm section of sperm with elongate acrosome (small arrow) penetrating egg jelly coat. Scale bar = 4.0 μm.

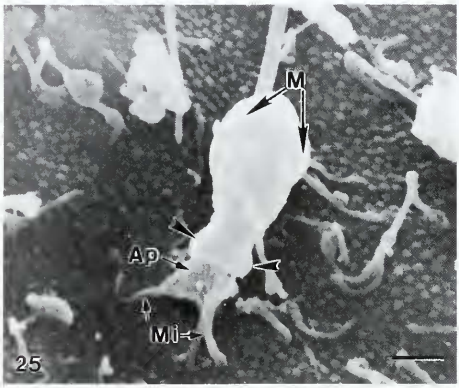
Figure 19. Transmission electron micrograph of a section of elongated anterior tip of acrosome (AT) in egg jelly coat (EJ). Note membranous vesicles (MV) in acrosome; posterior lobe (arrow) still protrudes into subacrosomal space, indicating lack of acrosome reaction. Scale bar = 0.4 μm. **Inset:** Microfilaments (small arrows) in elongated anterior tip of acrosome. Scale bar = 0.1 μm.

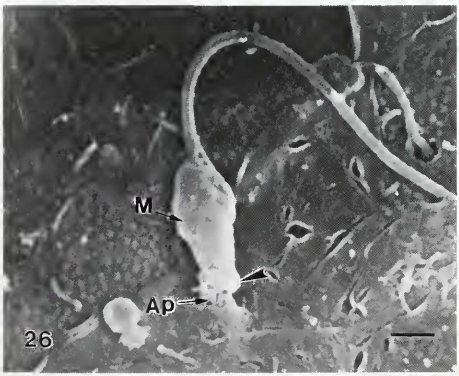












Figures 20–26.

Figure 20. Nomarski light micrograph of mature sperm of *Tectura scutum*, showing acrosome (A) nucleus (N) and mitochondrion (M). Scale bar = 2.0 μm.

Figure 21. Same sperm as in Fig. 20, labeled with anti-actin antibody conjugated to FITC, showing three regions of actin storage in the unreacted acrosome. Acrosome tip (a), anterior to subacrosomal space

Early events of fertilization

The acrosome of *T. testudinalis* appears to be involved in several key functions during fertilization, including (1) elongation of the anterior tip of the acrosome following contact with the egg jelly coat, (2) conversion of membrane precursors into new membrane to enable this elongation, (3) digestion of egg envelopes prior to sperm-egg fusion.

Acrosome elongation. Sperm association with the egg jelly coat triggers an elongation of the anterior tip of the acrosome in some sperm of *T. testudinalis*. This event is distinct from that in sea urchins and starfishes, where the jelly coat and its constituent compounds trigger an acrosome reaction (Epel, 1978; Hoshi *et al.*, 1994). Elongation of the anterior tip of the acrosome is distinct from an "acrosome reaction," as there is no eversion of the inner acrosomal membrane or protrusion of the subacrosomal space by an acrosomal process (Sato and Osanai, 1983). Micrographs of the anterior acrosome extension of *T. testudinalis* in the egg jelly coat suggest the presence of 6-nm microfilaments that were not apparent in the mature sperm before elongation. Azevedo made similar observations in *Patella lusitanica* (1981) and in the vetigastropod *Gibbula umbilicalis* (Azevedo *et al.*, 1985), reporting an extension of the anterior tip of the acrosome in response to egg water. Furthermore, in the related species *T. scutum*, we found actin localized at the tip, middle, and posterior of the acrosome (Figs. 20, 21). On the basis of these findings, we suggest that the newly formed microfilaments in the anterior tip of the acrosome of *T. testudinalis* are probably a result of polymerization of G-actin in the tip of the sperm acrosome, triggered by some property of the egg jelly coat. The mechanism of acrosome extrusion may vary among patellids, as evidenced by the report of pre-existing 9-nm filaments in the anterior acrosome of *Helcion* (Hodgson and Bernard, 1988). In other respects, the acrosome of these patellids is very similar to that of *T. testudinalis*.

We propose that the extension of the anterior tip of the acrosome has evolved to penetrate the egg jelly coat, when present. Although this elongation of the acrosome is not necessary for fusion of sperm and egg, it may provide a sperm with a selective advantage in penetrating the intact jelly coat of freshly spawned eggs. The egg jelly coat, besides functioning to stimulate extension of the acrosome in *Tectura*, may have other properties—including release of sperm chemoattractants (Miller, 1985) or species-specific sperm-activating agents (Tyler and Fox, 1940)—found in other patellids.

New membrane from precursors. The elongation of the acrosomal tip occurs within the limits of the plasma membrane. Consequently, any folds in the membrane are stretched out and the spherical mitochondria become compacted. Additional membrane may be developed from membrane precursors, as suggested previously for echinoderms (Dan, 1967; Colwin *et al.*, 1975; Sardet and Tilney, 1977; Tilney, 1985). Small membranous vesicles were found in elongating acrosomes in *T. testudinalis* but not in mature sperm prior to elongation. These vesicles apparently formed spontaneously during acrosome elongation and may be responsible for producing additional membrane by exocytosis. A very similar process, in which membranous vesicles formed spontaneously and contributed new membrane to the acrosomal process (Colwin *et al.*, 1975), was described in the fertilization of a holothuroid. Likewise, when sperm of *Gibbula umbilicalis* were exposed to seawater containing oocytes, several small vesicles were seen differentiating in the apical zone of the acrosome (Azevedo *et al.*, 1985).

The acrosome reaction and eversion of the inner acrosomal membrane. Following penetration of the egg jelly coat, sustained contact of the anterior tip of the acrosome with the vitelline layer overlying the small egg microvilli induces the acrosome reaction and subsequent fusion with the egg membrane. However, the larger, naked microvilli may supersede this process by reacting directly with the acrosome tip, or they may become involved subsequently by binding to the anterior portion of the acrosome.

(b), and subacrosomal space (c). Other sites of actin in the sperm are mitochondrial region (M) and flagellum. Scale bar = 2.0 μm.

Figure 22. Scanning electron micrograph showing extended acrosome tip (between arrowheads). Note position of nucleus (N) and posterior half of acrosome (PA). Scale bar = 1.0 μm.

Figure 23. Transmission electron micrograph of follicle cell containing sperm in heterophagic vacuoles in cytoplasm (arrows). Scale bar = 1.3 μm.

Figure 24. Scanning electron micrograph of sperm acrosome tip fusing with elongated egg microvilli (Mi). Note mitochondria (M). Scale bar = 1.0 μm.

Figure 25. Sperm acrosome reaction. Elongate egg microvilli (Mi), acrosome process (Ap), rolling back of plasma and outer acrosomal membranes (arrowheads), and compacted mitochondria (M). Scale bar = 1.0 μm.

Figure 26. Late stage of sperm entry through jelly coat, showing extended acrosomal process (Ap), rolling back of acrosome and plasma membranes (arrowhead), and compacted mitochondria (M). Scale bar = 1.0 μm.

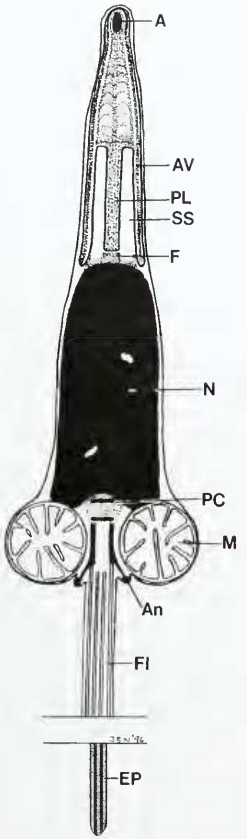


Figure 27. Diagram of mature sperm of *Tectura testudinalis*. Note: site of actin in acrosomal tip (A), acrosomal vesicle (AV), subacrosomal space (SS), posterior lobe (PL), flocculent material of subacrosomal space (F), nucleus (N), proximal centriole (PC), distal centriole and centriolar satellite connectives attached via annulus (An) to plasma membrane, mitochondrion (M), flagellum (Fl), and dense end-piece (EP) with reduced microtubules.

One key difference between *T. testudinalis* and other molluscan groups is the nature of the acrosomal process that extrudes the inner acrosomal membrane. In some vetigastropods, flocculent material located in the subacrosomal space is responsible for everting the inner acrosomal membrane by polymerization to a filamentous axial rod (Azevedo *et al.*, 1985). In other invertebrate species, including many echinoderms, arthropods, and annelids (see review by Dan, 1967), an acrosomal process is extruded from the subacrosomal space after polymerization of fibrogranular material into a microfilamentous rod. This contrasts with the mechanism observed in other molluscs—including some archaeogastropods (Lewis *et al.*, 1980) and bivalves (Tilney, 1985; Tilney *et al.*, 1987)—in which intersliding of actin filaments arranged in preformed rods causes extension of the acrosomal process.

Exactly how the inner acrosomal membrane everts in *T. testudinalis* is not known, but a similar polymerization of the flocculent fibrogranular material in the subacrosomal space by an actin-based mechanism is likely, considering that this site was one of three where actin was located in the related species, *T. scutum*. The final result is the extension of a short, broad acrosomal process, which contacts the egg microvilli and results in sperm-egg fusion. This type of acrosome reaction, as well as subsequent sperm entry into the egg, is very similar to that described for the polychaete *Tylorrhynchus heterochaetus* (Sato and Osanai, 1983; compare their fig. 6, p. 466, to our Fig. 25), as well as the scaphopod *Dentalium vulgare* (Dufrêsne-Dubé *et al.*, 1983) but is unlike the acrosome reaction and sperm entry described for other archaeogastropods (Lewis *et al.*, 1980; Shiroya and Sakai, 1984; Azevedo *et al.*, 1985).

Phylogenetics

In his analysis of gastropod phylogeny, Haszprunar (1988) contended, on the basis of characters such as gill structure, external fertilization with aquasperm, and symmetrical shell form, that docoglossan limpets (including Patellidae) are the basal offshoot of the ancestral gastropod. However, several authors have argued that almost none of these characters are primitive (see Ponder and Lindberg, 1996) and that in general "patellogastropods have few characters that match those seen in early Palaeozoic gastropod-like fossils" (Runnegar, 1996). Buckland-Nicks and Scheltema (1995) proposed that internal fertilization, not external, is basal to the Bilateria; evidence for this conclusion came from analysis of similar introsperm design in primitive members of vertebrate and invertebrate phyla. Among molluscs, the Neomeniomorpha, which have internal fertilization, are currently regarded as basal to the Mollusca in light of recent molecular and morphological analyses (Runnegar, 1996; Scheltema *et al.*, 1994). Thus, external fertilization does not necessarily denote primitive status among bilaterian phyla, although it is probably archetypal to the cnidarians and sponges.

Fertilization in limpets appears to be specialized in a number of ways, including the structure of the egg envelopes and the association with follicle cells; the use of a mechanism of sperm penetration that involves elongation of the acrosome tip prior to the acrosome reaction; and the expulsion of a short, broad acrosomal process. Furthermore, analysis of sperm basic proteins of limpets and

other gastropods indicated "a clear divergence of the protamines of *Patella* and other archaeogastropods" (Daban *et al.*, 1990, p. 127). This information and the above discussion suggest that it is premature to assign basal status to the Docoglossa. Rather, there is growing support for viewing limpets as a divergent group (Ponder and Lindberg, 1996) whose ancestors may have had internal fertilization with introsperm, a helicoid larval shell (Page, 1994), and primitive ctenidia.

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